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(54) Title: PROCESS FOR IDENTIFICATION OF GENES ENCODING PROTEINS HAVING CELL PROLIFERATION-PROMOTING ACTIVITY

(57) Abstract

The present invention is directed to selection systems for the identification of cell proliferation genes based on functional analysis. More specifically, the invention is directed to a process for the identification of a cell proliferation promoting activity, the isolation of genes involved in such cell proliferation promoting activity, and the use of the so identified genes for the diagnosis or treatment of a disease associated with excessive cell proliferation. The invention further is directed to the design and development of antibodies, peptides, nucleic acids, and other compounds which specifically interfere with the function of the identified gene and/or its gene product, and pharmaceutical compositions comprising such compounds, for the treatment of diseases associated with inappropriate or unregulated cell proliferation.

PROCESS FOR IDENTIFICATION OF GENES ENCODING PROTEINS HAVING CELL PROLIFERATION-PROMOTING ACTIVITY

I. FIELD OF THE INVENTION

The present invention relates to selection systems for the identification of novel cell proliferation genes. More specifically, the invention relates to a process for the identification of cell proliferation promoting activity, the isolation of genes involved in such cell proliferation promoting activity, and the use of the so identified genes for the diagnosis or treatment of a disease related to aberrant or unregulated cell proliferation. The invention further relates to the design and development of antibodies, peptides, nucleic acids, and other compounds which specifically interfere with the function or regulation of the identified gene and/or its gene product, and pharmaceutical compositions comprising such compounds, for the targeted treatment of diseases related to aberrant or unregulated cell proliferation.

II. BACKGROUND OF THE INVENTION

General Background. In the past decade it has become apparent that many diseases result from genetic alterations in signaling pathways. These include diseases related to unregulated cell proliferation such as cancers, atherosclerosis and psoriasis as well as inflammatory conditions such as sepsis, rheumatoid arthritis and tissue rejection. The finding that these proliferative diseases are based on genetic defects refocused the medical community to seek new modalities for disease management which essentially consist of designing drugs which modulate cell signaling. In order to develop highly specific drugs, *i.e.*, drugs which potently interfere with uncontrolled cell proliferation but have low toxicity or side effects, it is crucial to identify the genes encoding polypeptides involved in the cellular signal transduction pathways whose aberrant function may result in the loss of growth control.

Although tremendous progress in understanding relevant signal transduction pathways has been made in recent years, it is quite clear that many of the genes involved in the development of proliferative disorders, referred to herein generally as "cell proliferation genes", remain to be discovered.

Cell Proliferation Genes. Genes whose aberrant expression or function may contribute to cell proliferation disorders fall into two general categories: (1) dominant

Mutations in tumor suppressor genes and genes encoding products involved in the control of apoptosis are typically recessive; *i.e.*, both copies of the gene, the maternally inherited copy and the paternally inherited one, must be inactivated by mutation to remove the effect of the gene product. Usually, a single functional copy of such genes is sufficient to maintain tumor suppression. Predisposition to certain hereditary cancers involves mutant tumor suppressor genes. For example, if an individual inherits a single defective tumor suppressor gene from her father, initially her health will be uncompromised, since each cell still contains a functional copy of the gene inherited from her mother. However, as cells divide, mutations accumulate. Thus, at one point, the remaining normal copy in a cell may be inactivated by mutation to remove the function of the tumor suppressor, thereby completing one of the steps toward tumor formation. Such a cell may give rise to descendant cells which represent the early stages of cancer.

Of course, individuals who inherit a full normal complement of tumor suppressor genes can develop cancer as well. However, because two inactivating mutations are required, the development of the disease is much less frequent in such "normal" individuals, *i.e.*, not predisposed to cancer.

Tumor suppressor genes and oncogenes participate in growth control pathways in normal cells in such a way that the appropriate level of cell division is maintained. Disruption of these pathways by mutation of the component genes, oncogenes or tumor suppressor genes, is the underlying cause of cancer. Growth control in complex organisms like humans is a very important and complicated process. Thus, multiple genetic pathways for growth control are involved. Some pathways operate in all cell types in the body. Other pathways are much more specific and function only in certain cells.

Discovery Of Cell Proliferation Genes. Oncogenes and tumor suppressor genes have traditionally been identified by different methods. However, each of the approaches currently employed for the identification and isolation of cell proliferation genes has limitations on the types of genes that can be retrieved.

A first approach involves the detection and identification of transforming retroviruses and chromosomal translocations in tumors, which has provided the means to

Finally, a method which has been employed for isolating growth control genes of the tumor suppressor class involves the selection of variants that have lost certain malignancy traits, namely "revertants". Such revertant lines, however, are typically difficult to identify and separate from the majority of rapidly growing parental cells. Still, a number of such revertants have been isolated from populations of cells transformed by a variety of oncogenes and subsequent treatment with various cytotoxic agents which are toxic to growing cells or cancer cells. Fischinger *et al.*, 1972, *Science* 176:1033-1035; Greenberger *et al.*, 1974, *Virology* 57:336-346; Ozanne *et al.*, 1974, *J. Virol.* 14:239-248; Vogel *et al.*, 1974, *J. Virol.* 14:1404-1410; Cho *et al.*, 1976, *Science* 194:951-953; Steinberg *et al.*, 1978, *Cell* 13:19-32; Maruyama *et al.*, 1981, *J. Virol.* 37:1028-1043; Varmus *et al.*, 1981, *Cell* 25:23-26; Varmus *et al.*, 1981, *Virology* 108:28-46; Mathey-Prevot *et al.*, 1984, *J. Virol.* 50:325-334; Wilson *et al.*, 1986, *Cell* 44:477-487; Stephenson *et al.*, 1973, *J. Virol.* 11:218-222; Sacks *et al.*, 1979, *Virology* 97:231-240; Inoue *et al.*, 1983, *Virology* 125:242-245; Norton *et al.*, 1984, *J. Virol.* 50:439-444; Ryan *et al.*, 1985, *Mol. Cell. Biol.* 5:3477-3582. Usually, cells are exposed to these agents under such conditions where cells that have reacquired a non-transformed phenotype are contact inhibited, and hence, are less susceptible to these cytotoxic agents, leading to preferential elimination of the transformed parental cells and, after several cycles, the isolation of morphologic revertants.

In addition to being both inefficient and time consuming, the above described selection for tumor suppressor genes is based on differential growth parameters of normal versus transformed cells and hence may preclude the isolation of certain classes of revertants. Moreover, the selection procedure itself may induce epigenetic changes or changes in the number of chromosomes. Furthermore, if the cytotoxic agents used are themselves mutagenic, then their continuous presence during the selection period may generate a revertant phenotype resulting from multiple mutational events. While any of these mechanisms may result in the production of a revertant phenotype, the nature of these genetic or epigenetic changes may preclude their analysis by gene transfer experiments.

Obviously, the most constraining factor for the utility of tumor cells in gene discovery is the lack of powerful selection procedures allowing the identification of new

III. SUMMARY OF INVENTION

The subject invention is directed to selection systems for the identification of cell proliferation genes based on functional analysis. Generally, the selection procedures of the subject invention involve the use of variants of transformed cells to identify a cell proliferation promoting activity.

The selection systems of the invention may include creation of growth arrested tumor cell lines or cells which may undergo apoptosis, for example by the expression of a gene encoding a growth suppressor or apoptosis-inducing gene product, under the control of typically, an inducible promoter. When expression of the suppressor or apoptosis-inducing product is induced, growth of the tumor cells is suppressed and/or the cells die. Growth-proficient revertant cells are identified by virtue of their continued proliferation. Alternatively, if the efficiency of gene transfer is extremely high (as has been reported for certain retroviruses) and selection for cells that have taken up DNA is employed, regulated promoters can be eliminated. In this case, the tumor suppressor or apoptosis-inducing gene could be carried on the retrovirus along with a selectable marker such as hygromycin resistance. Revertants that express the selectable marker but do not die or undergo cell cycle arrest are then isolated directly.

The invention is further directed to the identification and isolation of genes involved in cell proliferation promoting activity. This may, for example, be accomplished by selecting spontaneous revertant cell lines, analyzing their gene expression pattern, and identifying differentially expressed genes.

In other embodiments, revertants are induced with specific molecules or moieties that disrupt a particular biochemical pathway, *i.e.*, "perturbagens". In one embodiment, the perturbagen is a DNA, encoding either a cell proliferation gene, or a protein or protein fragment acting akin to a dominant-negative mutant of cell proliferation genes, *e.g.*, by disruption of crucial protein/protein interactions. Revertants are selected, and the cell proliferation gene or protein/protein interaction underlying the promotion of cell growth can be determined by means of identification of the nature of the perturbagen. If the perturbagen is determined to be a cell proliferation gene, the corresponding gene product can be directly analyzed. If the perturbagen acts akin to a dominant-negative mutant, *e.g.*, by disrupting a protein/protein interaction in a signal transduction pathway,

Finally, the invention is directed to pharmaceutical compositions comprising such therapeutic compounds, and the use of such compositions for the treatment of diseases associated with aberrant or unregulated cell proliferation.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts a flow chart of the selection systems of the present invention for the identification of cell proliferation genes.

FIGURES 2A and 2B depict a flow chart exemplifying the use of perturbagens as a tool for the induction of revertants in the selection systems of the present invention for the identification of cell proliferation genes and protein/protein interactions.

FIGURES 3A and 3B depict the pOPRSVI.*p16* plasmid, a means of controlling *p16* tumor suppressor protein expression in cell lines.

FIGURE 4 depicts expression of *p16* in the revertant cell lines derived from HS294T/*p16*$^-$ cells.

FIGURE 5 depicts expression of *Rb* in the revertant cell lines derived from HS294T/*p16*$^-$ cells.

FIGURES 6A and 6B depict a peptide display and genomic fragment library vectors.

FIGURE 6A is a schematic representation of the peptide display library depicting the insertion point for 45 base oligonucleotides of the composition $(NNG/C/T)_{15}$ (N = any base) which encode randomized 15 amino acid peptides inserted in frame within GFP.

FIGURE 6B is a schematic representation of the genomic fragment expression library depicting the insertion point between the GFP coding region and the PGK1 3' UTR for small fragments of yeast genomic DNA (see Methods and Materials).

V. DEFINITIONS

As used herein, the following term(s), whether used in the singular or plural, will have the meanings indicated:

1995, *Cancer* 76:463-472. Because early detection and surgical resection play a vital role in survival rates, methods that facilitate early diagnosis are extremely important. One way to decrease the length of time between the appearance of tumor tissue and its detection is to survey candidate patients more frequently and more thoroughly. However, such methods of surveillance are expensive; thus it is necessary to limit scrutiny to high risk individuals. Consequently, information about genetic predisposition to cancer is extremely desirable. Because most genes that influence hereditary cancer are also involved in tumor progression, isolation of genes by somatic cell genetics has the potential to uncover such predisposing genes. Germline testing for such genes offers the chance to rate an individual's probability of contracting cancer, and expensive cancer screening efforts may be limited to those most likely to benefit from them.

Second, cell proliferation genes can be of medical value in the classification of already existing tumors based on genotype. Lowe *et al.*, 1994, *Science* 266:807-810. In the past, oncologists have relied on histological examination of biopsy specimens. Though useful, histological analyses are generally hampered by their subjectivity and imprecision. Methods that classify tumors based on their genetic composition have the potential to improve the reliability of their classification enormously. Detailed knowledge about tumor genotype may serve as a prognostic indicator for the tumor and may assist in guiding the therapeutic choice.

Finally, identification and isolation of cell proliferation genes affords important therapeutic opportunities. Numerous approaches may be pursued to use information about cell proliferation genes into therapies including, but not limited to the following: 1) transfer of wildtype tumor suppressor genes into tumor cells that have lost their activity; 2) inhibition of the activity of oncogenes in tumors, an approach that is being followed by several pharmaceutical companies in the development of *ras* farnesylation inhibitors; and 3) selective induction of tumor suppressor genes in normal cells to induce a state of temporary cell cycle arrest. These methods have the potential to be much more selective and efficacious than conventional chemo- or radiotherapy.

It is desirable to identify as many cell proliferation genes as possible because each one will be a candidate for medical utility.

Norton *et al.*, 1984, *J. Virol.* 50:439-444; Ryan *et al.*, 1985, *Mol. Cell. Biol.* 5:3477-3582; Zarbl *et al.*, 1991, *Environmental Health Perspectives* 93:83-89), the assays disclosed herein involve *positive* selection; *i.e.*, selection for growth, rather than the cessation of growth. It is easier to identify and separate growing cells from growth-arrested cells than to isolate non-transformed revertants.

Second, cultured tumor cell lines generally grow vigorously in culture. Thus, the assays of the invention can be performed in a time-efficient manner, as growing colonies can be identified, isolated, and analyzed very quickly.

Third, redundancy in growth control pathways is not a problem in the growth suppressed tumor cell lines provided and used for the selection systems of the invention, as is the case in assays based on selection for *non-transformed* cells. For example, in the case where a cell line is engineered to contain a gene encoding a wildtype tumor suppressor, one single restraint to growth remains. This growth restraint can be overcome by a variety of secondary changes, for example alterations in genes downstream of the particular tumor suppressor gene in the genetic pathway of growth control. Because of the fact that a single change can be sufficient to overcome the growth restraint of tumor suppressor-mediated arrest, methods that induce mutation (or perturbation) in a manner that allows recovery of the targeted gene in the cell permit isolation of additional cell proliferation genes. Accordingly, such cell proliferation genes are selected based on their inherent function as growth regulators in cells.

B. Selection Systems Based On Tumor Suppressor Genes

In one embodiment of the invention, selection systems are generated based on the growth suppression of tumor cell lines by the expression of a tumor suppressor gene, and proliferating revertants are selected.

1. Tumor Suppressor Genes

Many tumor suppressor genes cause growth arrest when overexpressed in normal cells, as well as in certain tumor cell lines. Examples for tumor suppressor genes include *p53* (Lin *et al.*, 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89:9210-4), *Rb* (Francke *et al.*, 1976, *Cytogenet. Cell Genet.* 16:131-134; Cavanee *et al.*, 1983,

In still another embodiment, selection systems are generated based on the breast cancer susceptibility tumor suppressor gene *BRCA1*. *BRCA1* has been shown to arrest growth of breast epithelial cell lines (Holt *et al.*, 1996, *Nat. Genet.* 12:298-302), however, little is known about *BRCA1's* pathway of growth control. Thus, selection systems based on BRCA1 suppressed tumor cells are of compelling interest and potential utility. Analysis of revertants of *BRCA1*-arrested cells, *e.g.*, in a *BRCA1*-overexpressing breast cancer cell line, *e.g.*, MCF7, can be used to identify downstream mediators of *BRCA1* tumor suppressor function.

In another embodiment of the invention, selection systems are designed based on the *p53* pathway. Regulated expression of *p53* and its downstream targets, such as the CDK inhibitor *p21* induces either apoptosis or G1 arrest in a variety of cell lines. Given the prominent role of *p53* in human cancer, *i.e.*, roughly 50% of human cancers contain *p53* mutations, information about other components of the *p53* pathway will be extremely valuable.

In still other embodiments of the invention, other tumor suppressor genes are used in order to design selection systems for the identification of novel cell proliferation genes. In principal, any gene whose expression can be manipulated to cause cell growth arrest, can be used. Examples include, but are not limited to, *WT1*, *VHL*, *BRCA2*, *NF1*, *NF2*, *P15*, *P21*, *P18*, *P19*, *P27*, *P57*.

2. Reversion Of Growth Arrested Phenotypes

Once arrested by expression of the tumor suppressor gene, revertant cells which continue to grow can be isolated.

In one embodiment of the invention, growth proficient random revertants are isolated. In other embodiments, reversion is induced using specific agents, *i.e.*, perturbagens, which are introduced into the growth suppressed target cell. *See, infra.*

Random Revertants. Generally, growth-proficient random revertants may proliferate for one of several reasons. First, they may have gained expression of an oncogene located downstream (or possibly upstream) of the tumor suppressor in the same genetic pathway. If this is the case, tumor genes can be directly identified. Second, the revertant cells may have undergone an alteration of a signaling pathway that is parallel to

growth control. Finally, the sixth cell line appeared to contain the expected levels of *p16* and *Rb* genes. The levels of the potential oncogenes CDK4 and cyclin D1, also thought to act in the *p16* growth control pathway, appeared normal as well. Thus, the sixth revertant cell line contained alterations in the expression or function of a gene of unknown identity. Based on its function, this gene is involved in the induction of the uncontrolled cell proliferation and thus possibly in the development of cancer. This cell line permits the identification of a novel cell proliferation gene.

Induced Revertants. In another embodiment of the invention, the identification of cell proliferation genes does not rely on the selection of random revertants. Growth-proficient revertants are induced using specific types of "mutagenic" agents, referred to as "perturbagens". Revertant cells are selected, and the gene or genes that allow escape from arrest are identified.

In one embodiment, the perturbagen is DNA encoding a cell proliferation gene, or, alternatively, dominantly active protein subdomains or peptide sequences, used to disrupt the action of endogenous tumor suppressors or oncogenes, *e.g.*, by interfering with crucial protein/protein interactions. Revertants are selected, and the cell proliferation gene or protein/protein interaction underlying the promotion of cell growth is determined by means of identification of the nature of the perturbagen.

If the perturbagen is determined to be a cell proliferation gene, it can be directly analyzed. For example, the perturbagen sequence is recovered using the Polymerase Chain Reaction (PCR) and sequenced using standard methods. If the perturbagen sequence is identical or similar to sequences in a public database such as GenBank or dbEST, then it can be directly identified. Alternatively, if a portion of the sequence is known, or even in the absence of any identification, the entire sequence of the perturbagen can be identified by isolating cDNA clones and standard recombinant DNA methodology.

The target of the perturbagen can be identified using a variety of methods. For example, if the perturbagen is acting akin to a dominant-negative mutant, *e.g.*, by disrupting a protein/protein interaction in a signal transduction pathway, the protein affected by the dominant-negative mutant is identified using assays suitable for the identification of protein/protein interactions, *e.g.*, the yeast two-hybrid system.

RNA that interferes with the stability or translation of specific cellular mRNAs. Most typically, such RNA-based perturbagens would act in an anti-sense manner by binding to complementary mRNA sequences in the cell.

Recovery and identification of the perturbagen sequences and their targets is accomplished with standard procedures, including the polymerase chain reaction (PCR) and the yeast two hybrid system. *See, Section VI.E., infra.*

The use of perturbagens for the induction of revertants in the selection systems of the present invention is depicted schematically in FIGURES 2A and 2B.

Once isolated, the perturbagen can be reintroduced into the same cell it was isolated from, or into different cell types to further characterize the properties of the molecule.

C. Other Selection Systems

CDK Inhibitors. In one embodiment of the invention, selection systems are generated based on expression of CDK inhibitors in suitable host cells.

All CDK inhibitors defined to date, including *p15, p16, p18, p19, p21, p27, p57* cause cell cycle arrest when they are overexpressed in certain cell lines. In some cases, such as *p16*, some details are alre idy known with respect to downstream pathway components. In other cases, most details of the pathway of growth control within which the genes function are still to be elucidated. Apart from their preferred *in vitro* targets, *i.e.*, CDK4 and CDK6 in the cases of *p15*, *p16*, and *p18*, and CDK4, CDK6, and CDK2 (and CDK4, CDK6) in the case of *p21*, *p27*, and *p57*, the identification of components of the pathways that act downstream by reversion selection systems will greatly facilitate the ability to manipulate these growth control pathways to achieve a therapeutic advantage.

Many cell lines respond to ectopic expression of CDK inhibitors by entering a state of arrest, and may be used for CDK inhibitor based selection systems accordingly. Exceptions are lines that have lost the activity of downstream mediators of the CDK inhibitor pathways. For example, *Rb*-cell lines cannot be forced into arrest by overexpression of *p16*. In addition, certain cell lines may have incurred mutations in downstream genes other than *Rb*. For instance, specific mutations in CDK4 render the

TABLE I

GENE	RECIPIENT CELL	EFFECT	REFERENCE
c-JUN	MCF7	inhibition of colony formation	Chen *et al.*, 1996, *Mol. Carcinog.* 15:215-226
EGF-R	Rat-1	inhibition of DNA synthesis	Daub *et al.*, 1996, *Nature* 379:557-560
GRB2	NIH3T3	inhibition of transformation	Xie *et al.*, 1995, *J. Biol. Chem.* 270:30717-30724
RAF	NIH3T3	inhibition of growth in soft agar	Denko *et al.*, 1995, *Somat. Cell. Mol. Genet.* 21:241-253
RAF	GH4	ras-induced promotor activation	Pickett *et al.*, 1995, *Mol. Cell. Biol.* 15:6777-6784
MAX	NIH3T3	natural growth regulation	Arsura *et al.*, 1995, *Mol. Cell. Biol.* 15:6702-6709
RAS	SK-N-MC	inhibition of ERK2 activation	van Weering *et al.*, 1995, *Oncogene* 11:2207-2214
SRC	endothelial	inhibition of c-FOS activation	Simonson *et al.*, 1996, *J. Biol. Chem.* 271:77-82

In principle, dominant negative proto-oncogenes can serve in the same way as tumor suppressor genes to arrest cells or prevent cell growth under certain conditions, thus providing a basis for selection of revertants.

Tumor Formation And Metastasis In Vivo. In another embodiment, selection systems are generated based on the observation that some tumor cell lines do not form tumors when injected into immunocompromised mice, while others do. For example, premalignant melanoma cell lines typically are nontumorigenic when placed in immunocompromised mice. In one embodiment, such premalignant melanoma cells are

Cells in culture can be induced to undergo apoptotic death by a variety of stimuli, depending on the particular cells. For example, certain cells enter apoptosis after exposure to glucocorticoids, tumor necrosis factors, or other natural agents. In addition, many cell types undergo apoptosis when exposed to radiation or chemotherapeutics. Further, cells may be engineered to contain genes which have been implicated in the control of or participation in apoptosis under the control of an inducible promoter. Such genes include, but are not limited to *bcl-2* (Korsymeyer, 1992, *Immunol. Today* 13:285-288), *c-myc* (Shi *et al.*, 1992, *Science* 257:212-214; Evan *et al.*, 1992, *Cell* 69:119-128), *p53* (Rotter *et al.*, 1993, *Trends Cell. Biol.* 3:46-49), *TRPM-2/SGP* (Kryprianou *et al.*, 1991, *Cancer Res.* 51:162-166), and *Fas/APO-1* (Itoh *et al.*, 1991, *Cell* 66:233-243). Cell types which can be induced to undergo apoptosis include, for example, lymphocytes and tumor cells derived from lymphocytes. Activation of the FAS antigen receptor in maturing lymphocytes activates an apoptosis program. If the FAS antigen is activated either by exogenous application of a FAS antibody (Velcich *et al.*, 1995, *Cell Growth Differ.* 6:749-757) or by ectopic expression of an activated form of the receptor, revertants that survive can be selected. Some of these revertants contain mutations in genes downstream of the FAS antigen that operate in the same apoptotic pathway as FAS. Treatment with certain steroid hormones or cross-linking of the T cell receptors on the cell surface using, for example, an antibody, can also induce apoptosis in lymphocytes and related cell or tumor lines. The 3DO line, for instance, responds to receptor cross-linking by undergoing apoptosis (Vito *et al.*, 1996, *Science* 271:521-525), while murine thymoma W7 cells undergo apoptosis in response to dexamethasone (Bourgeois *et al.*, 1993, *Mol. Endocrinol.* 7:840-851). Other cell lines undergo apoptosis when cultured at low density or in the absence of specific serum factors (Ishizaki *et al.*, 1995, *Mol. Endocrinol.* 7:840-851). In Friend erythroleukemia cells, overexpression of *p53* results in apoptosis (Abrahamson *et al.*, 1995, *Mol. Cell. Biol.* 15:6953-6960). Overexpression of certain oncogenes in some tumor lines can, paradoxically, also induce apoptosis (Harrington *et al.*, 1994, *Curr. Opin. Genet. Dev.* 4:120-129). The morphogen retinoic acid induces programmed cell death in the P19 embryonic stem cell (Okazawa *et al.*, 1996, *J. Cell Biol.* 132:955-968). It is also possible to use various forms of trauma to induce apoptosis in a variety of cell types. For instances, treatment of many cell types

tissue culture. In several cases the relevant factors have been defined. For example, in the absence of exogenous interleukin-2, certain T cells do not proliferate in culture. Melanoma formation proceeds via a series of steps through which normal melanocytes evolve into fully metastatic melanomas. During this process the progressing tumor cells gradually lose their requirements for specific exogenous factors (TABLE II). Normal melanocytes require factors such as phorbol ester, fibroblast growth factor (FGF), melanocyte stimulating hormone-alpha (MSH-α), insulin, or insulin-like growth factor-1 (IGF-1). In contrast, metastatic melanoma cells often require none of these factors. Cell lines with intermediate phenotypes require progressively fewer factors. This transition can be studied in culture such that factor-independent variants are isolated from earlier stage lines. These variants contain mutations that allow the cell to bypass the requirement for one of the factors. Thus, they can be used as the starting point for identification of genes that participate in the pathway of tumorigenesis involving escape from growth factor requirements.

they do not divide, but continue to produce growth factors that allow the T cell to proliferate into clone of descendants. This process of clonal growth can be used to select variants that are able to grow in the absence of a feeder layer.

Accordingly, revertant cells are selected that do grow at low density in colonies. These cells presumably contain alterations in genes involved in a pathway of growth dependence on neighbors, and hence, on secreted factors.

Immortalization. In still another embodiment, selection systems are generated based on the observation that normal cells, *e.g.*, primary mammalian cells, have a finite life span in culture; they undergo a certain number of cell doublings and then die. The length of their life in culture depends on a variety of factors including the tissue of origin, the age of the animal from which the cells were derived, and the nature of the growth media. The period during which massive cell death occurs as the cells reach their age limit is known as the crisis phase.

Accordingly, variants are selected that survive the crisis phase; these cells have undergone changes that lead to immortalization. In principle, this serves as a selection for immortalized cells with mutations in genes that normally limit life span.

D. Generation Of Growth Arrested Tumor Cell Lines As Selection Systems

Where the generation of the selection systems of the invention involves the expression of a growth suppressing or apoptotic gene in cultured cells, the nucleotide sequence encoding for the apoptosis regulator or inducer, or the growth suppressor, *e.g.*, a tumor suppressor gene or a dominant-negative oncogene or oncogene mutant, or a functional equivalent thereof, is inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence, and is introduced into the host cell system.

Expression Systems For The Expression Of The Growth Suppressing Genes. Typically, where a gene encoding the growth suppressor or the apoptosis-inducing product is introduced in a transformed cell, an inducible promoter system is used for the control of its expression. An inducible promotor permits growth of the cells that contain the expression construct under conditions where the promotor is turned off. When desired, the promotor can be induced and the cells become growth arrested due to the

product may require specific initiation signals for efficient translation of inserted cell proliferation gene encoding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the gene encoding the growth suppressor or the apoptosis-inducing product is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the cell proliferation gene encoding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. *See*, Bittner *et al.*, 1987, *Methods in Enzymol.* 153:516-544.

Though transient expression of the growth suppressor or the apoptosis-inducing product might be sufficient in some cases, most typically the gene encoding the growth suppressor or the apoptosis-inducing product will be stably expressed in the host cells. Host cells are transformed with DNA encoding the desired product controlled by appropriate expression control elements, including a promoter, which typically is inducible, *see*, *supra*, enhancer sequences, transcription terminators, polyadenylation sites, etc., and a selectable marker.

Following the introduction of foreign DNA, engineered cells are allowed to grow for 1-2 days in an enriched media, and then switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form colonies. The colonies are cloned and expanded into cell lines.

A variety of transfection techniques are currently available to transfer DNA *in vitro* into cells; including calcium phosphate-DNA precipitation, DEAE-Dextran transfection, electroporation, liposome mediated DNA transfer or transduction with recombinant viral or retroviral vectors, and may be used in the methods of the present invention.

encapsulate the helper viral RNA into particles is destroyed, and as a result only the recombinant retroviral vector containing a functional packaging signal and the gene of interest, but lacking the retrovirus' structural components can be incorporated in an particle. Consequently, the resulting retrovirus can infect a target cell, and its genetic information may be inserted into the host's genome; however, the so transferred genetic information is biologically contained because genes essential for viral growth are not provided. Methods for constructing and using retroviral expression systems are well known in the art and reviewed, for example, in Miller and Rosman, 1992. *Biotechniques* 7:980-990.

Identification Of Transfectants Or Transformants That Express The Growth Suppressing Or Apoptotic Gene. The host cells which contain the coding sequence and which express the biologically active gene product may be identified by at least four general approaches; (1) DNA DNA or DNA-RNA hybridization; (2) the presence or absence of "marker" gene functions; (3) assessing the level of transcription as measured by the expression of cell proliferation gene mRNA transcripts in the host cell; and (4) detection of the gene product as measured by immunoassay or by its biological activity.

In the first approach, the presence of the sequence encoding the desired product inserted in the expression vector is detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the sequence encoding the desired product, respectively, or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system is identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype. For example, if the gene encoding the growth suppressor or the apoptosis-inducing product is inserted within a marker gene sequence of the vector, recombinants containing the gene encoding the growth suppressor or the apoptosis-inducing product are identified by the absence of the marker gene function. Alternatively, a marker gene is placed in tandem with gene encoding the growth suppressor or the apoptosis-inducing product under the control of the same or different promoter used to control the expression of the sequence encoding the growth suppressor or the apoptosis-inducing product. Expression of the marker in response to induction or

revertant cell lines and hybridized in solution against mRNA from the other line. Shared sequences are removed from the cDNA probe by, for example, avidin-biotin capture, by binding to hydroxyapatite, or by any other suitable procedure. The remaining single stranded and thus unique cDNA sequences are then used to probe cDNA library filters to identify and isolate the differentially expressed sequences; alternatively, they may be cloned and examined directly. Hedrick *et al.*, 1984, *Nature* 308:149-153.

In still another embodiment, differentially expressed genes are detected by cloning and sequencing of high numbers of cDNA sequence fragments from the parent and revertant sources. Comparison of the sequences then leads to information about relative expression levels. This, for example, can be accomplished by sequence analysis of 3' expressed sequence tags (ESTs), a method pioneered by The Institute for Human Genome Research (TIGR) and by Human Genome Sciences, Inc. (HGS). Lennon *et al.*, 1996, *Genomics* 33:151-152. An alternative is to analyze small sequence tags cloned in multiple copies into plasmids or phage, a method known as Serial Analysis of Gene Expression (SAGE). Velculescu *et al.* , 1995, *Science* 270:484-487.

In still another embodiment polymerase chain reaction (PCR) is employed for identification of differentially expressed sequences, in an approach known as "differential display." The method takes advantage of the pseudo-random amplification that ensues when multiple primers of arbitrary sequences are placed in a reaction tube with random-primed cDNA. Certain fragments amplify and these are analyzed by denaturing gel electrophoresis. If two different cDNA samples are used separately, *i.e.*, one from a parental line, one from a revertant, the two PCR-amplified product sets can be run side-by-side on a gel. The intensities of different sized bands are compared, bands of different intensity are excised from the gel, reamplified and cloned for further analysis. Zhao *et al.*, 1995, *Biotechniques* 18:842-850.

In still other embodiments, gene expression is monitored and compared using protein levels as the output parameter. One method of differential protein analysis, for example, involves comparison of two-dimensional protein gels, whereby one dimension is non-denaturing, the second dimension is denaturing, to identify protein spots that are non-identically expressed in the two samples. Differences in samples of total protein isolates from the parental line are identified, the corresponding proteins are then purified

Biological Function And Relevance Of The Identified Cell Proliferation Gene And Its Product. The methods of the invention described above permit the identification of highly preselected candidates for crucial components of cellular growth proliferation pathways. In order to confirm their specific biological function and relevance, these candidates are tested in suitable *in vitro* and *in vivo* assays. The design of the assays will vary depending on the growth control pathway which was targeted by a particular selection system. For example, genes identified with selection systems based on, *e.g.*, the overexpression of a tumor suppressor may be expressed in cultured cells, *e.g.*, NIH3T3 cells, and their effect on cell growth, DNA synthesis, focus formation, growth in soft agar, modification, *e.g.*, phosphorylation of components or substrates in signal transduction pathways, complex formation of signal transduction components, including adapter molecules, changes in the pattern of gene expression, *e.g.*, induction of transcription factors, including *c-jun*, *c-fos*, *c-myc*, etc. is determined. *In vitro* assays are designed to determine substrate or ligand binding, phosphorylation signal transduction molecules, etc. Further, loss of function mutations may be generated in mice (knockout mice) or transgenic mice may be produced in which the gene is ectopically expressed. Dominant-negative mutants may be engineered in mouse or in human cells. Anti-sense constructs or oligonucleotides may be employed to downregulate expression of the specific gene. In certain cases, the gene or its homologs may be studied in yeast cells.

F. Expression Of The Cell Proliferation Gene In Cultured Cells

In order to express a biologically active cell proliferation gene in cultured cells, the nucleotide sequence encoding the cell proliferation gene, as identified and isolated as described in *Section VI.E.*, *supra*, or a functional equivalent, is inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. The cell proliferation gene products as well as host cells or cell lines transfected or transformed with recombinant cell proliferation gene expression vectors can be used for a variety of purposes. These include diagnostic uses, and generating antibodies (*i.e.*, monoclonal or polyclonal) that bind to the cell proliferation gene, as well as the identification of analogues or drugs that act on the cell proliferation gene, and for diagnostic purposes.

subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (*e.g.*, the 35S RNA promoter of CaMV; the coat protein promoter of TMV) are used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter) are used; when generating cell lines that contain multiple copies of the cell proliferation gene DNA SV40-, BPV- and EBV-based vectors are used with an appropriate selectable marker.

In bacterial systems a number of expression vectors are advantageously selected depending upon the use intended for the cell proliferation gene expressed. For example, when large quantities of cell proliferation gene product are to be produced for the generation of antibodies or to screen peptide libraries, vectors which direct the expression of high levels of fusion protein products that are readily purified are desirable. Such vectors include, but are not limited to, the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983, *EMBO J.* 2:1791), in which the cell proliferation gene coding sequence may be ligated into the vector in frame with the lacZ coding region so that a hybrid AS-lacZ protein is produced; pIN vectors (Inouye and Inouye, 1985, *Nucleic Acids Res.* 13:3101-3109; Van Heeke and Schuster, 1989, *J. Biol. Chem.* 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and are easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety.

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review, *see*, Current Protocols in Molecular Biology, Vol. 2, 1988, Eds. Ausubel *et al.*, Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant *et al.*, 1987, *Expression and Secretion Vectors for Yeast, in*: Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, *Heterologous Gene Expression in Yeast*, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152,

gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the cell proliferation gene in infected hosts. *See*, for example, Logan and Shenk, 1984, *Proc. Natl. Acad. Sci. U.S.A.* 81:3655-3659. Alternatively, the vaccinia 7.5K promoter may be used. *See*, for example, Mackett *et al.*, 1982, *Proc. Natl. Acad. Sci. U.S.A.* 79:7415-7419; Mackett *et al.*, 1984, *J. Virol.* 49:857-864; Panicali *et al.*, 1982, *Proc. Natl. Acad. Sci. U.S.A.* 79:4927-4931.

Specific initiation signals may also be required for efficient translation of inserted cell proliferation gene encoding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire cell proliferation gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the cell proliferation gene encoding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the cell proliferation gene encoding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. *See*, Bittner *et al.*, 1987, *Methods in Enzymol.* 153:516-544.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cells lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used.

Host cells containing the coding sequence and which express the biologically active cell proliferation gene product may be identified by several general approaches, including DNA-DNA or DNA-RNA hybridization, the presence or absence of "marker" gene functions, assessment of the level of transcription as measured by the expression of cell proliferation gene mRNA transcripts in the host cell, and the detection of the gene product as measured by immunoassay or by its biological activity. These approaches are described in more detail in *Section VI.D., supra.*

The cell proliferation gene products can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate transgenic animals using methods known in the art to introduce the cell proliferation associated transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Hoppe, P.C. and Wagner, 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten *et al.*, 1985, *Proc. Natl. Acad. Sci. U.S.A.* 82:6148-6152); gene targeting in embryonic stem cells (Thompson *et al.*, 1989, *Cell* 56:313-321); electroporation of embryos (Lo, 1983, *Mol. Cell. Biol.* 3:1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, *Cell* 57:717-723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, which is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry the cell proliferation associated transgene in all their cells, as well as animals which carry the transgene in some, but not all of their cells, *i.e.*, mosaic animals. The transgene may be integrated as a single transgene or in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko, M. *et al.*, 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89: 6232-6236). The regulatory sequences required for such a cell-type specific activation will depend upon the particular selected cell type, and will be apparent to those of skill in the art.

A variety of methods can be employed for the diagnostic and prognostic evaluation of diseases related to aberrant expression of cell proliferation associated genes, including cancer, and for the identification of subjects having a predisposition to such disorders. Such methods may, for example, utilize reagents such as the cell proliferation gene's nucleotide sequences described in *Section VI.E., supra*, and antibodies directed to the cell proliferation gene product, as described, in *Section VI.I., infra*. Specifically, such reagents may be used, for example, for: (1) the detection of the presence of cell proliferation gene mutations, or the detection of either over- or under-expression of the cell proliferation gene's mRNA relative to the state found in normal cell activation; (2) the detection of either an over- or an under-abundance of cell proliferation gene product relative to the normal state; and (3) the detection of perturbations or abnormalities in the signal transduction pathway mediated by the cell proliferation gene product.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one specific cell proliferation gene nucleotide sequence or antibody reagent directed to its gene product described herein, which may be conveniently used, *e.g.*, in clinical settings, to diagnose patients exhibiting cell proliferation disorder abnormalities.

For the detection of cell proliferation gene mutations, any nucleated cell can be used as a starting source for genomic or messenger nucleic acid. For the detection of the cell proliferation gene's expression or its gene products, any cell type or tissue in which the cell proliferation gene is expressed, most typically the afflicted tissue exhibiting a disease related to uncontrolled cell proliferation, may be utilized.

Nucleic acid-based detection techniques are described in *Section VI.G.1., infra*. Peptide detection techniques are described in *Section VI.G.2., infra*.

1. Detection Of The Cell Proliferation Gene And Its Transcript

In one embodiment, the cell proliferation gene cDNA or fragments thereof are used as a probe to detect the expression of the cell proliferation gene mRNA. For example, sections of tissue samples may be prepared and examined by *in situ* hybridization with a suitable, labelled probe. Alternately, mRNA extracts may be

reaction (PCR). PCR is described in detail in U.S. Patents 4,965,188, 4,683,195, and 4,800,195.

In still other embodiments, mutations within the cell proliferation gene can be detected by utilizing a number of techniques. Nucleic acid from any nucleated cell can be used as the starting point for such assay techniques, and may be isolated according to standard nucleic acid preparation procedures which are well known to those of skill in the art.

DNA may be used in hybridization or amplification assays of biological samples to detect abnormalities involving gene structure, including point mutations, insertions, deletions and chromosomal rearrangements. Such assays include, but are not limited to, Southern analyses, single stranded conformational polymorphism analyses (SSCP), and PCR analyses.

Diagnostic methods for the detection of cell proliferation gene-specific mutations can involve for example, contacting and incubating nucleic acids including recombinant DNA molecules, cloned genes or degenerate variants thereof, obtained from a sample, *e.g.*, derived from a patient sample or other appropriate cellular source, with one or more labeled nucleic acid reagents including recombinant DNA molecules, cloned genes or degenerate variants thereof, under conditions favorable for the specific annealing of these reagents to their complementary sequences within the cell proliferation gene. Preferably, the lengths of these nucleic acid reagents are at least 15 to 30 nucleotides. After incubation, all non-annealed nucleic acids are removed from the nucleic acid molecule hybrid. The presence of nucleic acids which have hybridized, if any, is then detected. Using such a detection system, the nucleic acid from the cell type or tissue of interest can be immobilized, for example, to a solid support such as a membrane, or a plastic surface such as that on a microtiter plate or polystyrene beads. In this case, after incubation, non-annealed, labeled nucleic acid reagents are easily removed. Detection of the remaining, annealed, labeled cell proliferation gene's nucleic acid reagents is accomplished using standard techniques well-known to those in the art. The cell proliferation gene sequences to which the nucleic acid reagents have annealed is compared to the annealing pattern expected from a normal gene sequence in order to determine whether a gene mutation is present.

alternatively, to test the effect of compounds on the expression of the cell proliferation gene.

For example, antibodies, or fragments of antibodies useful in the present invention, such as those described in *Section VI.I., infra*, may be used to quantitatively or qualitatively detect the presence of the cell proliferation gene products or conserved variants or peptide fragments thereof. This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently labeled antibody (*see*, this Section, *infra*) coupled with light microscopic, flow cytometric, or fluorimetric detection.

The antibodies (or fragments thereof) or fusion or conjugated proteins useful in the present invention may, additionally, be employed histologically, as in immunofluorescence, immunoelectron microscopy or non-immuno assays, for *in situ* detection of the cell proliferation gene products or conserved variants or peptide fragments thereof, or for catalytic subunit binding (in the case of labeled catalytic subunit fusion protein).

In situ detection may be accomplished by removing a histological specimen from a patient, and applying thereto a labeled antibody or fusion protein of the present invention. The antibody (or fragment) or fusion protein is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the cell proliferation gene product, or conserved variants or peptide fragments, but also its distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Immunoassays and non-immunoassays for cell proliferation gene products or conserved variants or peptide fragments thereof will typically comprise incubating a sample, such as a biological fluid, a tissue extract, freshly harvested cells, or lysates of cells which have been incubated in cell culture, in the presence of a detectably labeled antibody capable of identifying the cell proliferation gene products or conserved variants or peptide fragments thereof, and detecting the bound antibody by any of a number of techniques well-known in the art.

spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect the cell proliferation gene product through the use of a radioimmunoassay (RIA) (*see*, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are

method known in the art for the synthesis of DNA and RNA molecules. For example, oligonucleotides may be synthesized chemically using commercially available DNA or RNA synthesizers like machines sold by Applied Biosystems. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which comprise suitable RNA polymerase promoters such as the T3, T7, or the SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, may be introduced stably into cell lines.

Various modifications to the DNA and RNA molecules may be introduced as a means of increasing the intracellular stability and half-life. For example, flanking sequences of ribo- or deoxy- nucleotides may be added to the 5' and/or 3' ends of the molecule, or phosphorothioate or 2' O-methyl rather than phosphodiester linkages may be used within the oligonucleotide backbone. Xu *et al.*, 1996, *Nucleic Acid Res.* 24:1602-1607.

I. Generation And Use Of Cell Proliferation Gene Antibodies

Various procedures known in the art may be used for the production of antibodies to epitopes of the recombinantly produced cell proliferation genes identified and isolated employing the selection systems of the present invention. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library. Such antibodies may be useful, *e.g.*, as diagnostic or therapeutic agents. As therapeutic agents, neutralizing antibodies, *i.e.*, those which compete for binding with a ligand, substrate or adapter molecule, or interfering with the cell proliferation genes activity, are of especially preferred interest.

For use as diagnostic agents, monoclonal antibodies that bind to the cell proliferation gene are radioactively labeled allowing detection of their location and distribution in the body after injection. Radioactivity tagged antibodies may be used as a non-invasive diagnostic tool for imaging *in vivo* the presence of a tumors and metastases associated with the expression of said cell proliferation gene.

Antibody fragments which contain specific binding sites of the cell proliferation gene may be generated by known techniques. For example, such fragments include, but are not limited to, $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse *et al.*, 1989, *Science* 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to the cell proliferation gene.

J. Use Of Revertant Cells Or The Isolated Cell Proliferation Genes For The Identification Of Compounds Useful For The Treatment Of Disease Related To Uncontrolled Cell Proliferation

1. Identification Of Compounds

The revertant cells identified using the selection system process of the invention, may be used directly, *i.e.*, without isolation of the relevant cell proliferation gene, for the identification and isolation of compounds inhibiting aberrant cell proliferation. Alternatively, the cell proliferation genes identified by the process of the invention may be isolated and used for *in vitro* or *in vivo* assays for the identification and isolation of compounds specifically interfering with their activity.

More specifically, the identified revertant cells may be exposed to chemical compounds or compound libraries, and compounds exhibiting growth inhibition may be identified. Alternatively, the identified cell proliferation genes may be expressed in suitable expression systems, designed to allow for high-throughput testing of compounds from any source to identify molecules having an inhibitory effect on the cell proliferation genes.

Nucleotide sequences encoding the cell proliferation genes identified and isolated using the selection systems of the invention may be used to produce the corresponding purified protein using well-known methods of recombinant DNA technology. Among the many publications that teach methods for the expression of genes after they have been isolated is Gene Expression Technology, Methods and Enzymology, Vol.:185, edited by Goeddel, Academic Press, San Diego, California (1990).

In one embodiment of the invention, the cell proliferation genes and/or expressing cell lines expressing the cell proliferation gene are used to screen for antibodies, peptides, organic molecules or other ligands that act as agonist or antagonists of the cell proliferation gene activity. For example, antibodies capable of interfering with the activity, *e.g.*, enzymatic activity of the cell proliferation gene, or with its interaction with a ligand, adapter molecule, or substrate are used to inhibit the cell proliferation gene function. In cases where amplification of the cell proliferation gene function is desired, antibodies which mimic, *e.g.*, a ligand, an adapter molecule or substrate of the corresponding the signal transduction pathway may be developed. Obviously, if desired, antibodies may be generated which modify the activity, function, or specificity of the cell proliferation gene.

Alternatively, screening of peptide libraries or organic compounds with recombinantly expressed cell proliferation gene protein or cell lines expressing the cell proliferation gene may be useful for identification of therapeutic molecules that function by inhibiting, enhancing, or modifying its biological activity.

Synthetic compounds, natural products, and other sources of potentially biologically active materials can be screened in a number of ways. The ability of a test compound to inhibit, enhance or modulate the function of the cell proliferation gene may be determined with suitable assays measuring the cell proliferation gene function. For example, responses such as its activity, *e.g.*, enzymatic activity, or its ability to bind its ligand, adapter molecule or substrate may be determined in *in vitro* assays. Cellular assays may be developed to monitor a modulation of second messenger production, changes in cellular metabolism, or effects on cell proliferation. These assays may be performed using conventional techniques developed for these purposes. Finally, the ability of a test compound to inhibit, enhance or modulate the function of the cell proliferation gene will be measured in suitable animal models *in vivo*. For example, mouse models will be used to monitor the ability of a compounds to inhibit the development of solid tumors, or effect reduction of the solid tumor size.

In one embodiment of the invention, random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support are used to identify peptides that are able to interfere with the function of the cell proliferation gene.

As an alternative to whole cell assays for membrane bound receptors or receptors that require the lipid domain of the cell membrane to be functional, the receptor molecules can be reconstituted into liposomes where a label or "tag" can be attached.

In another embodiment, cell lines that express the cell proliferation gene or, alternatively isolated cell proliferation gene protein or fragments thereof, are used to screen for molecules that inhibit, enhance, or modulate the cell proliferation gene activity or signal transduction. Such molecules may include small organic or inorganic compounds, or other molecules that effect the cell proliferation gene activity or that promote or prevent the complex formation with its ligand, adapter molecules, or substrates. Synthetic compounds, natural products, and other sources of potentially biologically active materials can be screened in a number of ways, which are generally known by the skilled artisan.

For example, the ability of a test molecule to interfere with the cell proliferation gene function may be measured using standard biochemical techniques. Alternatively, cellular responses such as activation or suppression of a catalytic activity, phosphorylation or dephosphorylation of other proteins, activation or modulation of second messenger production, changes in cellular ion levels, association, dissociation or translocation of signalling molecules, or transcription or translation of specific genes may also be monitored. These assays may be performed using conventional techniques developed for these purposes in the course of screening.

Further, effects on the cell proliferation gene function may, via signal transduction pathways, affect a variety of cellular processes. Cellular processes under the control of the cell proliferation gene signalling pathway may include, but are not limited to, normal cellular functions, proliferation, differentiation, maintenance of cell shape, and adhesion, in addition to abnormal or potentially deleterious processes such as unregulated cell proliferation, loss of contact inhibition and, blocking of differentiation or cell death. The qualitative or quantitative observation and measurement of any of the described cellular processes by techniques known in the art may be advantageously used as a means of scoring for signal transduction in the course of screening.

Various technologies may be employed for the screening, identification, and evaluation of compounds which interact with the cell proliferation genes of the invention.

out its disruptive activity, and would therefore be preselected for membrane permeability, which is a desirable or even crucial pharmacological property. Moreover, the screen has general applicability since it can be used against any protein-protein interaction which can be recapitulated within a cell. Furthermore, the screen is efficient because the cells can be gridded out in wells into which compounds are applied, either individually or in pools, and the reporter construct can be assayed independently in each well. The assay might consist of a colorimetric output to report the presence or absence of the interaction, which may be performed *in vivo* or *in vitro*, or an *in vivo* cell growth assay.

Generally, in a first step, the protein-protein interaction is determined or verified, and in a second step, inhibitors of the interaction are identified.

Assays For The Identification And Determination Of Protein-Protein Interactions. Any method suitable for detecting protein-protein interactions may be employed for identifying intracellular proteins that interact with the cell proliferation gene product. Among the traditional methods which may be employed are co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns of cell lysates or proteins obtained from cell lysates to identify proteins in the lysate that interact with the cell proliferation gene product. For these assays, the cell proliferation gene product used can be a full length gene product, or a truncated peptide. Once isolated, such an interacting protein can be identified and can, in turn, be used, in conjunction with standard techniques, to identify proteins with which it interacts. For example, at least a portion of the amino acid sequence of an intracellular protein which interacts with cell proliferation gene product, can be ascertained using techniques well known to those of skill in the art, such as via the Edman degradation technique. (See, *e.g.*, Creighton, 1983, "Proteins: Structures and Molecular Principles", W.H. Freeman & Co., N.Y., pp.34-49). The amino acid sequence obtained may be used as a guide for the generation of oligonucleotide mixtures that can be used to screen for gene sequences encoding such intracellular proteins. Screening may be accomplished, for example, by standard hybridization or PCR techniques. Techniques for the generation and screening of oligonucleotide mixtures are well-known. (See, *e.g.*, Ausubel, supra., and PCR Protocols: A Guide to Methods and Applications, 1990, Innis, M. et al., eds. Academic Press, Inc., New York).

product. By way of example, and not by way of limitation, the cell proliferation gene product may be used as the bait gene product. Total genomic or cDNA sequences are fused to the DNA encoding an activation domain. This library and a plasmid encoding a hybrid of a bait cell proliferation gene product gene product fused to the DNA-binding domain are cotransformed into a yeast reporter strain, and the resulting transformants are screened for those that express the reporter gene. For example, and not by way of limitation, a bait cell proliferation gene sequence, such as the open reading frame of the cell proliferation gene product or a domain thereof, is cloned into a vector such that it is translationally fused to the DNA encoding the DNA-binding domain of the *GAL4* protein. These colonies are purified and the library plasmids responsible for reporter gene expression are isolated. DNA sequencing is then used to identify the proteins encoded by the library plasmids.

A cDNA library of the cell line from which proteins that interact with bait cell proliferation gene product are to be detected can be made using methods routinely practiced in the art. According to the particular system described herein, for example, the cDNA fragments can be inserted into a vector such that they are translationally fused to the transcriptional activation domain of *GAL4*. This library can be co-transfected along with the bait cell proliferation gene product gene-*GAL4* fusion plasmid into a yeast strain which contains a lacZ gene driven by a promoter which contains *GAL4* activation sequence. A cDNA encoded protein, fused to a *GAL4* transcriptional activation domain, that interacts with bait cell proliferation gene product will reconstitute an active *GAL4* protein and thereby drive expression of the *HIS3* gene. Colonies which express *HIS3* can be detected by their growth on petri dishes containing semi-solid agar based media lacking histidine. The cDNA can then be purified from these strains, and used to produce and isolate the bait cell proliferation gene-interacting protein using techniques routinely practiced in the art.

Small Molecule Displacement Assay To Identify Inhibitors Of The Protein-Protein Interaction. The macromolecules that interact with the cell proliferation gene product are referred to, for purposes of this discussion, as "binding partners". These binding partners are likely to be involved in the cell proliferation gene product signal transduction pathway, and therefore, in the role of the cell proliferation gene product's

of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction by competition can be identified by conducting the reaction in the presence of the test substance; *i.e.*, by adding the test substance to the reaction mixture prior to or simultaneously with the cell proliferation gene product and interactive binding partner. Alternatively, test compounds that disrupt preformed complexes, *e.g.*, compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a heterogeneous assay system, either the cell proliferation gene product or the interactive binding partner, is anchored onto a solid surface, while the non-anchored species is labeled, either directly or indirectly. In practice, microtiter plates are conveniently utilized. The anchored species may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the cell proliferation gene product or binding partner and drying. Alternatively, an immobilized antibody specific for the species to be anchored may be used to anchor the species to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the partner of the immobilized species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (*e.g.*, by washing) and any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the non-immobilized species is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the non-immobilized species is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; *e.g.*, using a labeled antibody specific for the initially non-immobilized species (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds which inhibit complex formation or which disrupt preformed complexes can be detected.

can be performed, if desired, to ensure that the library sequence is indeed the binding partner (*i.e.*, the target) of the perturbagen *in vivo*.

Once the perturbagen and its target are identified, it is possible to reconfigure the two-hybrid interaction so that screens for small molecules can be undertaken. Such screens take advantage of the protein-protein interaction between the perturbagen and its target. They seek out small molecules that are capable of displacing the protein-protein interaction. Technically, such a screen could be carried out in yeast cells, in mammalian cells in which the interaction has been reconstituted, or, perhaps best of all, in a test tube. Such a screen is configured by fusing one of the binding partners (e.g., the perturbagen) to a convenient reporter molecule such as Green Fluorescent Protein (GFP). The other binding partner (e.g., the target) is fused to a second protein that can be absorbed onto a solid support via a biotin bridge or an antibody or some other ligand. The interaction between the perturbagen and its target must be maintained in the new fusion setting. The release of GFP fluorescence signal from the solid support (i.e., into the supernatant) is then detected after addition of test compounds. Compounds that are able to displace the GFP/perturbagen fusion are candidates for perturbagen mimics. Some of these may bind the perturbagen, while others may bind the target. These two classes are readily distinguished by subsequent tests with the perturbagen and the target.

In general, the displacement assay must utilize a reporter construct in the cell that is not too sensitive to distances or geometries between the two protein partners. It can be applied in numerous different cell systems, a few examples are described in the following.

Yeast. The traditional two-hybrid system in yeast may be applied in both the *GAL4* version and the *lexA* formulation. Bartel *et al.*, 1995, *Methods Enzymol.* 254:241-263; Mendelsohn *et al.*, 1994, *Curr. Opin. Biotechnol.* 5:482-486. Both systems take advantage of the bipartite nature of yeast transcription factors. The DNA binding component can be separated from the activation component and each fused to different proteins. If the proteins interact strongly enough with each other, a functional transcription factor is reconstituted and the reporter gene(s) are turned on. In the *GAL4* version, the reporters are *HIS3* and *lacz*. These genes are engineered to contain GAL4 binding sites upstream in a suitable position to provide activation if and only if an

a. Indications For The Use Of Compounds Interfering With The Cell Proliferation Genes Of The Invention

The compounds identified by the methods of the present invention are modulators of a cell proliferation activity in general, or a cell proliferation gene in particular. As such, the compounds produced by the processes and assays of the invention are useful for the treatment of disease related to aberrant, uncontrolled or inappropriate cell proliferation.

A large number of disease states involve excess or diminished cell proliferation. Generally, many of these diseases may be treated with DNA sequences, proteins, or small molecules that influence cell proliferation. In some instances the goal is to stimulate proliferation; in others, to prevent or inhibit proliferation of cells. The list of diseases directly involving cell growth includes, but is not limited to, cancer, psoriasis, inflammatory diseases, such as rheumatoid arthritis, restenosis, immunological activation or suppression, including tissue rejection, neurodegeneration or expansion of neuronal cells and viral infection.

Accordingly, pharmaceutical compositions comprising a therapeutically effective amount of a compound identified by the methods of the invention will be useful for the treatment of diseases driven by unregulated or inappropriate cell proliferation, including cancer, such as glioma, melanoma, Kaposi's sarcoma, psoriasis, hemangioma and ovarian, breast, lung, pancreatic, prostate, colon and epidermoid cancer, rheumatoid arthritis, psoriasis, restenosis, immunological activation or suppression, including tissue rejection, neurodegeneration or expansion of neuronal cells.

K. Formulations/Route Of Administration

The identified compounds can be administered to a human patient alone or in pharmaceutical compositions where they are is mixed with suitable carriers or excipient(s) at therapeutically effective doses to treat or ameliorate a variety of disorders. A therapeutically effective dose further refers to that amount of the compound sufficient to result in amelioration of symptoms as determined in a decrease of cell proliferation. Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

obtained as a solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients include fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration,the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be

A pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase.

The cosolvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic compounds well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, *e.g.*, polyvinyl pyrrolidone: and other sugars or polysaccharides may be substituted for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually with a greater toxicity.

Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days.

Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (*See, e.g.*, Fingl *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the kinase modulating effects, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from *in vitro* data; *e.g.*, the concentration necessary to achieve 50-90% inhibition of the kinase using the assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using MEC value. Compounds should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%. In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

4. Packaging

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labelled for treatment of an indicated condition. Suitable conditions indicated on the label may include inhibition of cell proliferation, treatment of a tumor, treatment of arthritis, and the like.

As a consequence, the cells, termed HS294T/*p16*$^-$, respond to IPTG by induction of *p16* and cell cycle arrest. Death of arrested HS294T/*p16*$^-$ cells after addition of IPTG occurred over a period of several days during the second week of arrest. By day fifteen (15), no viable cells were present and the vast majority of the adherent cells had disappeared from the bottom of the culture dish.

B. Example 2: Selection Of Growth-Proficient Revertants

In this example, the selection of growth-proficient generated as revertants derived from the growth suppressed H2594T/*p16*$^-$ melanoma cells generated as described in Example 1, *supra*, is described. Further analysis of the revertants will reveal the identity of cell proliferation genes useful for the diagnosis and prognosis of diseases related to uncontrolled or inappropriate cell proliferation, and for the development of targeted drugs for the treatment of disease related to uncontrolled cell proliferation.

To select revertants from the population of *p16*-arrested cells, HS294T/*p16*$^-$ cells were plated in microtiter wells at a density of 2000 cells/well in the presence of IPTG. As a control, parental HS294T cells that continue to grow in the presence of IPTG were seeded at different densities among arrested HS294T/*p16*$^-$ cells in a separate set of microtiter wells. As expected, these wells gave rise to growing clones of cells that spread over the well bottom.

By day twenty (20) after plating, 11/96 microtiter wells clearly contained growing cells. Assuming that a single progenitor cell spawned the colony in each of the eleven wells, this implies a reversion rate of approximately one per 20.000 arrested cells (11/96(2000)).

Materials And Methods. The melanoma cell line HS294T was engineered to contain an IPTG-inducible *p16* gene in the PopRSV vector (Stratagene, San Diego, CA) as described by Stone *et al*, 1996, *Cancer Res.*, in press. The resulting cell line, HS294T/*p16*, was arrested by addition of 0.1mM IPTG after subculturing 2,000 cells per well of a 96-well culture plate (Falcon). Fresh medium (DMEM, nonessential amino acids, glutamine (2 mM), sodium pyruvate (100 mg/ml), hygromycin (30 μg,ml), geneticin (34 μg/ml), IPTG (0.1 mM) was added every four (4) to five (5) days. After twenty (20) days, eleven (11) wells were judged to contain growing cells, six (6) of

TABLE III

Line	G_1/G_2 (-IPTG)	G_0/G_1 (+IPTG)
POP	2.6	2.7
POP/p16	3.5	45.0
rev1	3.5	6.6
rev2	2.2	3.1
rev3	3.6	4.0
rev4	1.9	1.2
rev5	3.8	4.4
rev6	1.7	1.8

The expression status of the *p16*, *Rb*, and *CDK4* gene products was examined in the revertant line by western blot analysis. *See*, FIGURES 4 and 5. Four of the six lines had lost expression of the inducible *p16* construct. A fifth line had no detectable *Rb* protein, while a sixth line, *rev6*, appeared to have the expected levels of *p16*, *Rb*, *CDK4* and cyclinD1.

Flow Cytometry. Revertant and control cell lines were grown to about 70% confluency and treated with 0.1 mM IPTG for twenty four (24) hours. The cells were immediately harvested, fixed in ethanol, and stained with propidium iodide prior to analysis on a FACscan flow cytometer (Becton-Dickinson). Estimates of cells in G_1 and G_2 were made by fitting Gaussian curves to the fluorescence data and integrating the curves using the program (Modfit; Verity House Software).

Western Blots. The revertant and control cell lines were treated with 0.1 mM IPTG for twenty four (24) hours prior to making total cell lysates. $1x10^7$ cells were washed and resuspended in lysate buffer (0.1 M NaCl, 0.01 M TrisCl pH 7.6, 1 mM EDTA pH 8.0), boiled, and frozen at -80°C. Approximately equal amounts of thawed total protein were run on SDS polyacrylamide gels and transferred using the semi-dry method (Hoeffer) onto nitrocellulose membranes. Blocking and antibody treatment of the blots was according to standard procedures (BioRad). Primary antibodies were obtained from various sources: anti-*p16*, anti-CDK4, and anti-cyclin-D1 where obtained from the ICRF (London, UK); anti-RB was obtained from Santa Cruz Biotechnology

A large-scale screen was carried out for both random peptides and fragments of yeast genomic DNA that cause escape from α-factor-induced cell cycle arrest. Fourteen different fragments of yeast genomic DNA and two randomly generated peptides were identified which, when expressed, promoted escape from cell cycle arrest. Of the fourteen (14) genomic fragments, nine (9) are predicted to encode portions of yeast proteins, including portions of the STE11 and STE50 proteins, two genes involved in the pheromone-response pathway. Hartwell, 1980, *J. Cell. Biol.* 85:811-822; and Rad *et al.*, 1992, *Mol. Gen. Genet.* 236:145-154. The remaining five (5) fragments are predicted to express relatively short peptides not found in any known or predicted yeast coding sequence. Thus, genetic screens employing perturbagen libraries represent an effective means for identifying genes involved in important cellular processes. In addition, for pathways relevant to human diseases such as cancer, perturbagen-based genetic methods may lead to novel therapeutic agents and targets.

Strains And Media. The *Saccharomyces cerevisiae* strains used in the screen for α-factor-resistant colonies was yVT12 (MATa, ura3-1, leu2-3, 112, lys2, sst2Δ, hmla, hmra, mfa1Δ::hisG, mfa2::hisG, ade2-1, STE::GAL1-STE3::HIS3, strain JRY5312. Boyartchuk *et al.*, 1997, *Science* 275:1796-1800. Yeast strains were transformed by the method of Gietz and Schiestle (Gietz and Schiestl, 1995, *Methods in Molecular and Cellular Biology* 5:255-269f) and plasmids were maintained by growth in standard media. Isolation of plasmids from yeast was accomplished by harvesting cells from 2 ml overnight cultures by centrifugation, discarding the supernatant, and resuspending cells in 200 µl of extraction buffer (2% Triton; 1% SDS; 100 mM NaCl; 10 mM Tris, pH 8; and 1 mM EDTA). 200 µl of phenol:chloroform (1:1) equilibrated with TE (100 mM Tris, pH 8; and 10 mM EDTA) and a small volume of 425-600 micron acid-washed glass beads (Sigma) were then added and the mixture vortexed for one (1) to two (2) minutes. Organic and aqueous phases were separated by centrifugation and the aqueous phase removed and extracted with phenol. 1 µl of the aqueous phase was used to transform DH5-α *E. coli* cells by electroporation.

genomic fragment libraries were introduced into yeast strain yVT12 by standard techniques generating yeast libraries of $3x\ 10^7$ and $1x\ 10^7$ transformants, respectively.

Methods. The constructs employed for the generation of the peptide display library are depicted in FIGURE 6A. In brief, 45 residue oligonucleotides of the composition $(NNG/T/C)_{15}$ were inserted into pVT21 using XhoI and BamHI restriction enzyme sites which had previously been engineered into the green fluorescent protein (GFP) at nucleotide position 468. The yeast genomic fragment library was constructed by digesting genomic DNA from strain yVT5 (MATa, leu2-3, 112, trp1-1, ura3-1, his3-11, 15, ade2-1, can1-100 [strain JRY2334] with DpnII (New England Biolabs, Beverly, MA), isolating digested DNA 100-500 base pairs in length from a 1% agarose gel using the gene clean III kit (BIO 101), and ligating the purified DNA to pVT21 that had previously been digested with BglII (New England Biolabs, Beverly, MA), treated with calf intestinal phosphatase (New England Biolabs, Beverly, MA), and purified. Following ligation, DNA was introduced into *E. coli* strain DH5-α by electroporation, and the resulting amplified library purified using the Qiagen "maxi-prep" kit (Quiagen, San Diego, CA).

The library size was estimated *via* serial dilutions of the primary *E. coli* transformations. The average size of library inserts was determined by isolating DNA from twenty (20) individual library colonies and determining insert sizes by restriction digest analysis.

2. Identification Of Library Clones That Confer α-Factor Resistance

To identify library clones that promoted resistance to α-factor-induced cell cycle arrest, a primary screen for α-factor-resistant colonies was carried out. Aliquots containing on average about two (2) to three (3) yeast cells of each primary transformant derived from both the peptide and genomic fragment libraries were grown for six (6) hours in rich media containing galactose and raffinose as carbon sources (YEPGR) in order to induce expression of the library plasmids. Following this induction, cells were spread onto 150 x 15 mm YEPGR plates ($1x\ 10^6$ cells per plate) containing $1x\ 10^{-8}$ M α-factor, which is the minimal concentration of α-factor required to arrest yVT12 cells at this plating density. *See, infra.* 1750 α-factor-resistant colonies

densities of 1-5x 10^2 and 1x 10^5 cells on 100 x 15 mm plates were 5x 10^{-10} M and 1x 10^{-8} M, respectively. For 1x 10^6 cells plated on a 150 x 15 mm plate, 1-5x 10^{-8} M α-factor was required and concentrations between 10^{-7} M and 10^{-6} M were required to arrest thicker patches transferred by replica plating. Minimal concentrations of α-factor required to arrest cells were the same on both YEPGR and YEPD plates.

TABLE IV

Strength of Cell Cycle Arrest Escape Phenotypes

Library Plasmid Number[a]	Number of Colonies Per Plate[b]				Percentage of Cells Escaping from αFactor Arrest	
	YEPD		YEPGR			
	-αF	+αF	-αF	+αF	Dextrose	Galactose
pVT21	204	0	100	0	<.005	<.01
1	242	1	158	155	.004	98
2	432	1	262	158	.002	60
3	302	0	116	62	<.003	53
4	231	0	240	47	<.004	20
5	420	0	376	23	<.002	6
6	400	0	240	146	<.003	61
7	386	0	64	29	<.003	45
8	412	0	382	42	<.002	11
9	500	0	376	30	<.002	8
10	366	0	ND	34	<.003	ND
11	696	0	404	57	<.001	14
12	936	4	449	47	.009	10
13	440	0	444	278	<.002	54
14	696	0	227	77	<.001	34

a. The numbers 1-14 refer to each of the fourteen (14) perturbagen plasmids, pVT21 is the parental vector for the library.

b. Colonies counts were performed five (5) days after the initial plating.

822; Rhodes *et al.*, 1990, *Genes and Dev.* 4:1862-1874; Rad *et al.*, 1992, *Mol. Gen. Genet.* 236:145-154; and Xu *et al.*,1996, *Molec. Microbiol.* 20:773-783. Indeed, overexpression of either the N-terminal half of the protein encoded by the STE11 gene or a C-terminal truncation allele of STE50 (ste50-2), both of which are similar to the regions overexpressed in these two library clones, have been previously reported to decrease sensitivity to pheromone to varying degrees (Stevenson, 1992, *Genes and Dev.* 6:1293-1304; and Rad *et al.*, *supra*. Thus, one class of perturbagen molecules that can be identified in such broad screens are portions of proteins that are themselves directly involved in the process under study. Examination of the roles played by the proteins encoded by six (6) of the seven (7) remaining ORFs (Numbers 6, 8, and 10-14) in the pheromone-response pathway, and the identification of the targets inhibited by all the perturbagens, may resolve how often perturbagens themselves are portions of proteins involved in the process under study, and how wide the potential range of perturbagen targets is.

PCR Amplification And Sequencing Of Library Clone DNA. Whole-colony PCR was performed by transferring yeast cells from single colonies to PCR vessels, microwaving the cells for one minute at full power, and immediately cooling the cells on ice. After cooling, PCR reactions were performed using standard reagents and protocols. Ausubel *et al.*, (eds) *Current Protocols in Molecular Biology*, John Wiley and Sons, New York (1996). Primers used to amplify the genomic inserts were oVT201 (5'-ATT TTA GCG TAA AGG ATG GGG-3'), which is homologous to a region within the PGK1 3' untranslated region (3'UTR), and oVT326 (5'-TGA GAA TTC GGA TCC AAG AGA GAC CAC ATG GTC C-3'), part of which is homologous to a region within the GFP coding region. Sequencing of the 5' and 3' ends of genomic inserts present in both PCR-amplified products and plasmid DNA was accomplished with primers oVT326 and oVT201, and sequence data was obtained using an ABI373A DNA sequencer (Applied Biosystems Division, Perkin-Elmer, Inc., Foster City, CA).

the strength of the perturbagens in a simple colony formation assay. To determine the penetrance of each perturbagen plasmid, yeast harboring either one of each of the fourteen (14) perturbagen plasmids or the parental vector pVT21 were grown overnight to mid-log phase in selective liquid media containing either glucose or galactose/raffinose as a carbon source. 250 μl of dilutions containing 1000 cells/ml of each overnight culture were then plated onto either YEPGR or YEPD media (depending on the carbon source present in the media in which they were grown) that either contained or lacked 5x 10^{-10} M α-factor (the lowest concentration of α-factor necessary to arrest strain yVT12 at this cell density). Colonies on the various plates were counted after five days and the fractions of the total number of cells plated in the presence of α-factor able to form colonies for each plasmid were determined. The results of this analysis clearly reveal differences in the strength of the perturbagens. Some have penetrance of 100% in the assay; others are less than 10% penetrant. The basis for these different penetrances is not clear. It may involve differences in Ki's among the various perturbagens, differences in their expression levels, and/or differences among their targets.

E. Example 5: Selection Systems Based On Expression Of The *Retinoblastoma* Gene Product

In analogy to the *p16*-arrest experiments, the *rb* gene may be expressed in tumor cells to select for the identification of novel cell proliferation genes. The so obtained selection systems may be used for the selection of random revertants or for the isolation of revertants obtained upon induction with perturbagens. *See, supra.* Revertants of *rb*-arrested cells are expected contain alterations in a set of genes that overlaps considerably with the *p16*-arrested revertants because *rb* acts downstream in the same signal transduction pathway as *p16*.

Further analysis of the *rb*-revertants will reveal the identity of cell proliferation genes useful for the diagnosis, prognosis, and for the development of targeted drugs for the treatment of diseases related to unregulated or inappropriate cell proliferation related to the *rb* signal transduction pathway.

the treatment of diseases related to unregulated or inappropriate cell proliferation associated with CDK inhibitors.

I. Example 9: Selection Systems Based On Components Of Oncogene Pathways

In order to identify the components of oncogene pathways, dominant-negative oncogenes or oncogene fragments of interest are expressed ectopically in a transformed cell such that growth is inhibited or apoptosis is induced. The dominant-negative negative oncogenes and cell systems employed in this experiment are listed in TABLE I, *supra*. The transformed cell lines may be used for the selection of random revertants or for the isolation of revertants obtained upon induction with perturbagens.

Revertant cells are isolated and analyzed to identify altered proliferation genes downstream in the oncogene's growth control pathway. These proliferation genes may be useful for the diagnosis, prognosis and for the development of targeted drugs for the treatment of diseases related to unregulated or inappropriate cell proliferation associated with oncogenes.

J. Example 10: Selection Systems Based On Tumor Formation And Metastasis *In Vivo*

Genes that render tumorigenic cells non-tumorigenic are overexpressed in tumor cell lines. The non-tumorigenic cells are injected into immuno-compromised mice, *e.g.*, nude mice, followed by the isolation of clonal tumor variants. Revertant cell lines may be induced by introduction of perturbagens.

Analysis of these revertant cells permits the isolation of important cell proliferation genes that contribute to tumor formation, and genes that contribute to tumor formation *in vivo* may be directly analyzed and recovered. The so obtained genes may be used for the diagnosis, prognosis, and for the development of targeted drugs for the treatment of diseases related to unregulated or inappropriate cell proliferation associated with aberrant expression or control of these cell proliferation genes.

Reversion of the cells may also be induced by introduction of perturbagens. The mutations that have eliminated the function of the regulatory pathway that prevents growth in the absence of the factor are identified and the corresponding genes recovered. The so obtained genes can have numerous medical applications, including diagnosis and prognosis of diseases related to uncontrolled cell proliferation, and the development of drugs for the treatment of such diseases.

N. Example 14: Selection Systems Based On The Inability Of Non-Transformed T-Cells To Grow In Isolation

Many non-transformed T-cell lines can only be cloned, *i.e.*, grown in isolation from other neighbors when the individual cells are placed on a "feeder layer" of other cells.

Non transformed T cell lines are diluted to a concentration of 100 cells/ml; 10 ml of this cell suspension are then seeded on a ten (10) cm tissue culture plate. Revertants cells which do grow at low density in colonies are selected. Such revertants are presumed to contain alterations in genes involved in a pathway of growth dependence on neighbors, and hence, depend on secreted factors. Revertant cells are selected and isolated, and the corresponding genes are recovered. The so identified cell proliferation genes may be used, *e.g.*, for diagnosis, prognosis, and the development of targeted drugs for cancer therapy.

O. Example 15: Selection Systems Based On Immortalization Of Primary Cells

Freshly isolated human primary epithelial cells are cultured in suitable media; the vast majority of the cells has a finite lifespan and die after a certain number of cell doublings. Revertants which survive the "crisis phase" are selected. These revertant cells have undergone changes that lead to immortalization and contain for mutations in genes that normally limit life span. Subsequently, the differentially expressed or initiated genes from these revertant cells when compared to normal primary cells are recovered.

CLAIMS

WHAT IS CLAIMED IS:

1. A process for the identification of genes encoding a product having a cell proliferation-promoting activity comprising identifying within a plurality of growth arrested transformed cells revertant transformed cells that are not growth arrested.

2. The process of Claim 1 wherein growth arrest of said transformed cells is caused by expression of a tumor suppressor gene in said transformed cells.

3. The process of Claim 2 wherein said tumor suppressor gene has been introduced on an expression plasmid under the control of a promoter.

4. The process of Claim 3 wherein the tumor suppressor gene is expressed under the control of an inducible promoter.

5. The process of Claim 4 wherein the tumor suppressor gene is selected from the group consisting of *P16*, *P53*, *RB1*, *WT1*, *BRCA1*, *BRCA2*, *NF1*, *NF2*, *P15*, *P18*, *P19*, *P21*, *P27*, *P57* and *VHL*.

6. The process of Claim 5 wherein the tumor suppressor gene is *p16*.

7. The process of Claim 4 wherein the transformed cells correspond to a transformed cell line selected from the group consisting of lines derived from primary tumors, metastatic tumors, transformed primary cells and immortalized primary cells.

8. The process of Claim 7 wherein the transformed cell line is expressing *rb*.

9. The process of Claim 8 wherein the transformed cell line is a melanoma cell line.

18. The process of Claim 17 wherein said cell component is selected from the group consisting of a cell proliferation gene and a gene product thereof.

19. The process of Claim 18 wherein the perturbagen is selected from the group consisting of a DNA encoding a peptide, a DNA encoding a polypeptide, and a peptide.

20. The process of Claim 15 wherein the perturbagen is a retroviral sequence randomly inserted in the transformed cell's genome.

21. The process of Claim 20 wherein the perturbagen disrupts the activity of a cellular tumor suppressor gene, or its downstream target(s) said process further comprising:

(a) identifying said tumor suppressor gene; and

(b) isolating said tumor suppressor gene.

22. The process of Claim 20 wherein the perturbagen disrupts the activity of a cellular tumor oncogene, or its downstream target(s) said process further comprising:

(a) identifying said cellular oncogene; and

(b) isolating said cellular oncogene.

23. A process for the identification of pharmaceutical compounds for the treatment of a disease associated with aberrant cell proliferation by inhibiting the growth of cells comprising:

(a) incubating a revertant cell obtained by the process of Claim 1 with test compounds; and

(b) determining from said test compounds candidate compounds which promote inhibition of proliferation of said revertants cells when compared to a control cell.

31. A method for treating a disease associated with excessive cell proliferation comprising administration of an effective amount of the pharmaceutical composition of Claim 29.

32. A method for treating a disease associated with excessive cell proliferation comprising administration of an effective amount of the pharmaceutical composition of Claim 30.

33. A method for treating a disease according to Claim 31 wherein the disease is selected from the group consisting of cancer, arteriosclerosis, psoriasis, and rheumatoid arthritis, retenosis, any condition involving cell proliferation.

34. A method for treating diseases according to Claim 32 wherein the disease is selected from the group consisting of cancer, arteriosclerosis, psoriasis, and rheumatoid arthritis.

35. A method to identify expression of a cell proliferation gene obtained by the process of Claim 13, 16, 18, 21, or 22 in a tissue sample comprising:

(a) exposing nucleic acid derived from the mRNA of said tissue sample to a labelled oligonucleotide probe having a sequence substantially complementary to a fragment of said cell proliferation gene; and

(b) identifying hybridization of said oligonucleotide probe with said nucleic acid under stringent conditions.

36. An antibody against the cell proliferation gene produced by the process of any of Claims 13, 16, 18, 21, or 22.

37. A method to identify expression of a cell proliferation gene produced by the process of any of Claims 13, 16, 18, 21, or 22 in a tissue sample comprising:

contacting a tissue sample from a patient with an antibody against the cell proliferation gene.

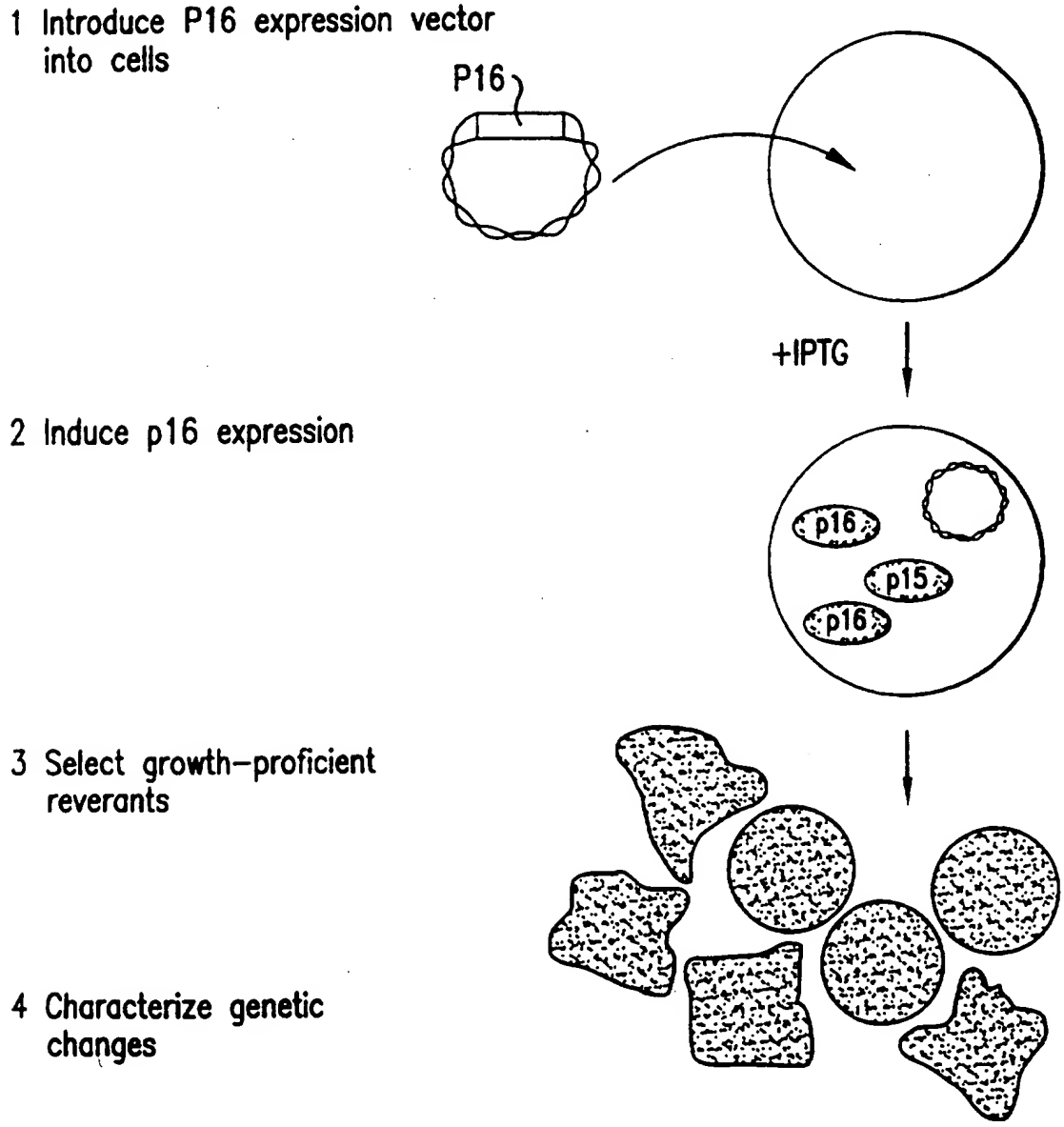
1 Introduce P16 expression vector into cells
P16
+IPTG
2 Induce p16 expression
p16
p15
p16
3 Select growth-proficient reverants
4 Characterize genetic changes

FIG.1

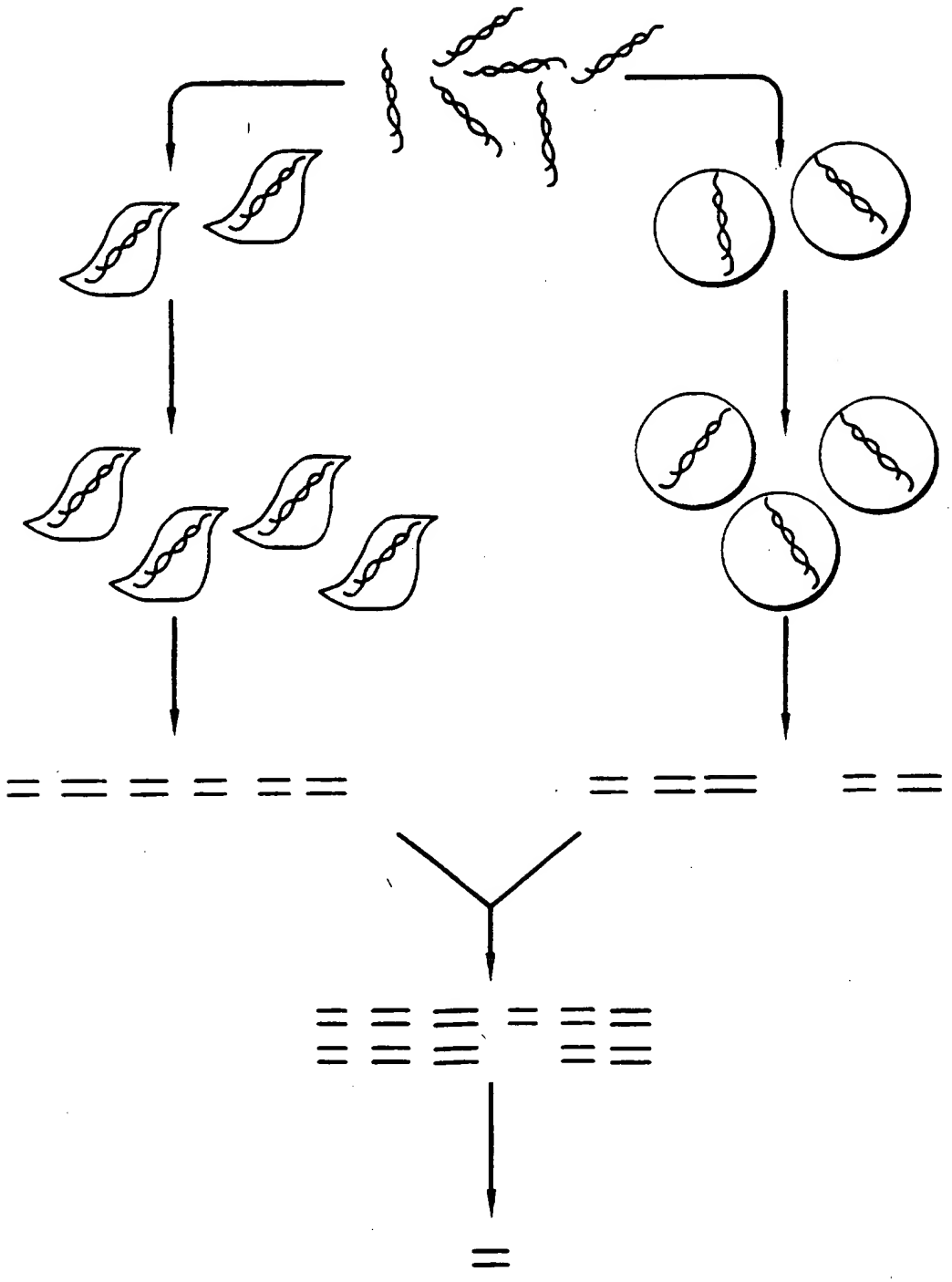

FIG.2B

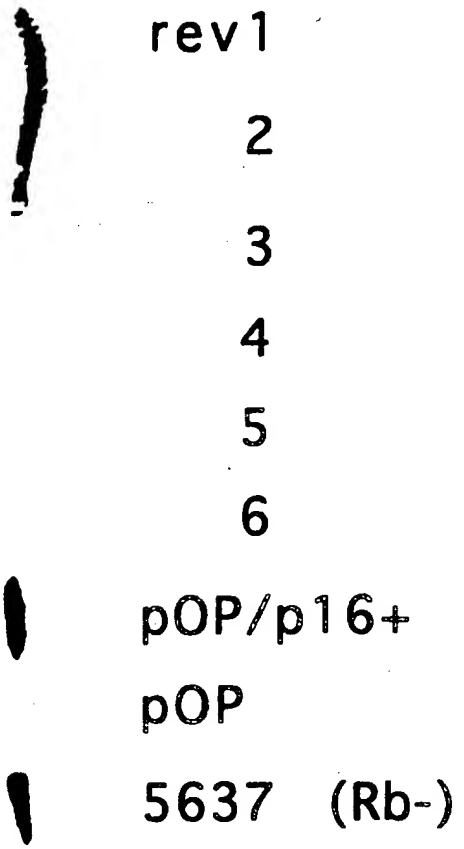

FIG.4

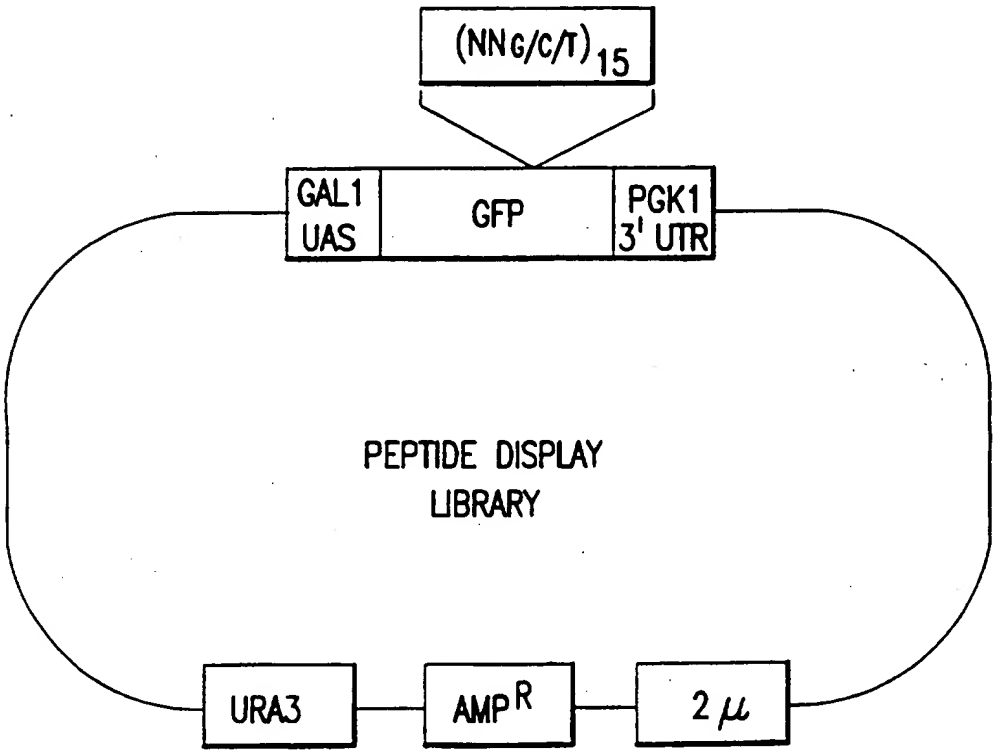
(NNG/C/T)15
GAL1 UAS
GFP
PGK1 3' UTR
PEPTIDE DISPLAY LIBRARY
URA3
AMPR
2μ

FIG.6A

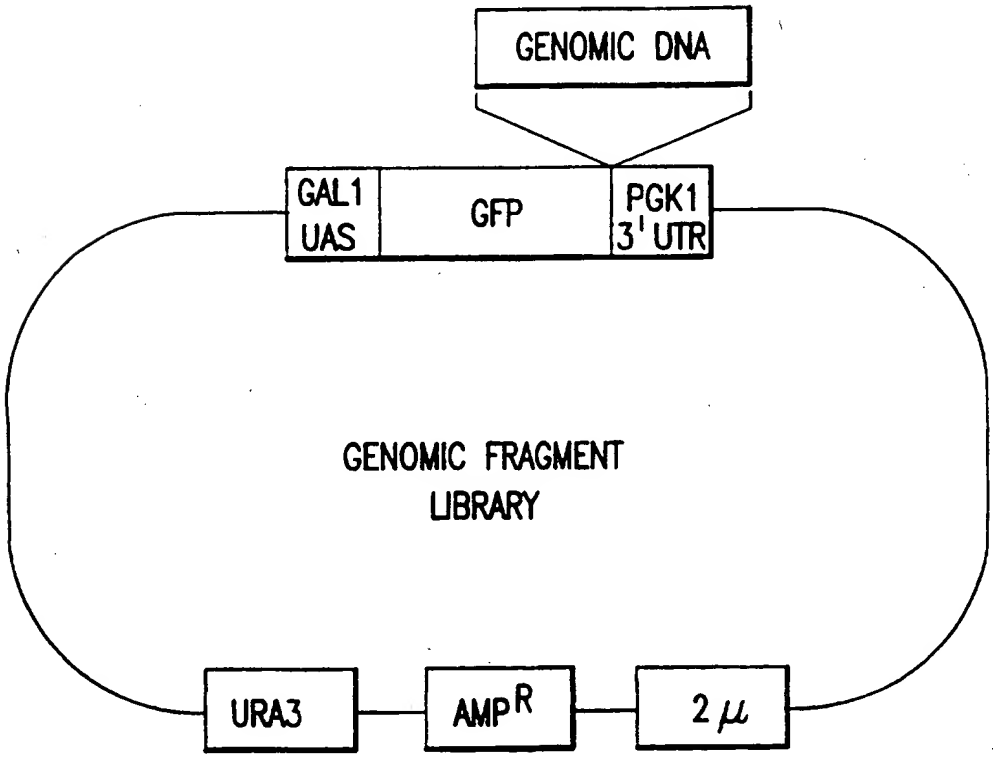
GENOMIC DNA
GAL1 UAS
GFP
PGK1 3' UTR
GENOMIC FRAGMENT LIBRARY
URA3
AMPR
2μ

FIG.6B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14514

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HIRANO et al. Roles of *p53* Mutation in Cell Line Establishment and Identification of the Minimum Transactivation and Transform Suppression Domains. Oral Oncol. Eur. J. Cancer. 1995, Vol. 31B, No. 2, pages 129-135, see entire document.	1-3
X	CHEN et al. Activation and Inhibition of the AP-1 Complex in Human Breast Cancer Cells. Mol. Carcinogenesis. 1996, Vol. 15, pages 215-226, see pages 222-224.	1-3, 14-19
X	BISHOP et al. Cellular Oncogenes and Retroviruses. Ann. Rev. Biochem. 1983, Vol. 52, pages 301-354, especially pages 306-308.	1, 2, 13-22

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAPLUS, MEDLINE, BIOSIS, EMBASE, INPADOC, SCISEARCH, LIFESCI
search terms:
melanoma, HS294T, p53, tumor, transformed, cancer, rb, p16, revertant

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-22, 35 and 38, drawn to processes for gene identification.

Group II, claim(s) 23-25, 28-34, drawn to pharmaceutical compounds, methods of making and methods of using said compounds.

Group III, claim(s) 26 and 27, drawn to assays for compound identification using polypeptide.

Group IV, claim(s) 36 and 37, drawn to antibodies and method of use.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I does not share a special technical feature with groups II-IV because the claimed processes for gene identification lack the special technical features of disease treatment, pharmaceutical identification or raising antibodies. Likewise, group II does not share a special technical feature with groups III and IV because the methods of pharmaceutical identification of group II lack the special technical feature of polypeptide expression. Finally, the antibodies and methods of use of group IV does not share a special technical feature with any of the other groups because they involve the special technical features of antigen preparation, antibody generation and diagnosis with the antibodies obtained. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.